Simvastatin decreases lipopolysaccharide induced pulmonary inflammation in healthy volunteers

Murali Shyamsundar, Scott TW McKeown, Cecilia M O'Kane, Thelma R Craig, Vanessa Brown, David R Thickett, Michael A Matthay, Cliff Taggart, Janne T Backman, J Stuart Elborn, Daniel F McAuley

Online Data Supplement

Methods

Subjects

Exclusion criteria included creatinine kinase (CK) greater than 5 times the upper limit of the normal range; alcohol abuse or abnormal liver function tests with transaminases greater than 3 times the upper limit of the normal range; previous history of asthma; pregnancy, breast-feeding or women of childbearing potential not using adequate contraception; previous or concurrent use of statins, concomitant use of drugs known to interact with simvastatin or any abnormality on screening.

LPS (Escherichia coli serotype O26:B6; Sigma Chemicals, Poole, Dorset, UK) was dissolved in endotoxin free sterile 0.9% saline and inhaled via an automatic inhalation–synchronized dosimeter nebuliser (Spira, Hameenlinna, Finland). This delivers particles of a mass median aerodynamic diameter (MMAD) of 10 μm as described previously (1). The dosimeter produces a calibrated aerosol of 8 μl at each slow inhalation starting from functional residual capacity (FRC) to total lung capacity (TLC). Each subject performed five successive inhalations of the LPS solution (1.25 mg/mL) through the mouthpiece with a nose clip in place. The total dose of inhaled LPS was 50 μg. This dose has previously been demonstrated to induce a local alveolar and systemic inflammatory response (2, 3).

Bronchoalveolar lavage (BAL) was performed 6 hours after LPS inhalation according to standard guidelines (4). Three successive 60 ml aliquots of 0.9% saline were instilled into a subsegment of the right middle lobe and each aspirated immediately with low suction.

Bronchoalveolar lavage fluid (BALF) return was measured and immediately placed on ice until transferred to the laboratory for processing. BAL fluid was centrifuged at 900g for 5 minutes at 4°C. The supernatant was removed and stored at –80^{o}C for subsequent analysis. The cell pellet was re-suspended in 10ml phosphate buffered saline (PBS) supplemented with 1% bovine serum albumin (BSA) (Sigma Chemicals). Total cell count was determined using a haemocytometer. Cell viability was measured by the ability of live cells to exclude Trypan blue. Cytospins were prepared and stained with Speedy-Diff (Clin-Tech Ltd, UK). Differential cell count was performed by a single cytologist independent of the research team thus maintaining blinding.

Plasma samples

Blood was collected on day 4 prior to the subject taking the final dose of study drug under supervision (1 hour prior to LPS inhalation) and at 24 hours after LPS inhalation and placed immediately on ice until processed. Blood was centrifuged at 3000g for 10 minutes at 4°C The plasma was removed and stored at –80^{o}C for subsequent analysis.

Determination of plasma simvastatin and simvastatin acid.

The concentrations of simvastatin and simvastatin acid in plasma were measured by liquid chromatography-ion spray tandem mass spectrometry using the PE Sciex API 2000 LC/MS/MS system (Sciex Division of MDS Inc, Toronto, Ontario, Canada) as previously described (5). Simvastatin-D6, and simvastatin acid-D6 served as internal standards. The limit of quantification was 0.05 ng/ml for simvastatin and 0.05 ng/ml for simvastatin acid. The interday coefficients of variation were for simvastatin 7.9% at 0.1

ng.ml-1 and 2.1% at 50 ng/ml (n=6) and for simvastatin acid 15.1% at 0.1 ng/ml and 1.5% at 50 ng/ml (n=6).

Flow Cytometric Analysis

BAL cells were gated on a granulocyte population using a forward scatter and side scatter plot. Negative controls consisted of omission of AV and PI, as positive controls are not readily accessible for apoptosis detection. To eliminate cellular debris from the analysis, the discrimination level was set at 100. A minimum of 10,000 cells were analyzed for each sample. Analysis of samples was performed using the Immuno 4 software programme which involved subtraction of negative controls from positive samples. Due to technical difficulties or research staff experienced in the use of flow cytometry not being available it was only possible to measure neutrophil apoptosis in 9 of 10 subjects in the placebo group and 15 of 20 subjects in the statin group respectively. Flow cytometry was performed as previously described (6).

Nuclear Factor-κB (NFkB) measurement

Monocytes were isolated from peripheral blood from healthy volunteers by centrifugation across a Ficoll-Paque gradient and adherence as previously described (7). $1x10^7$ mononuclear cells were plated per 100mm dish and allowed to mature for 6 days in RPMI/10% FCS and 10ng/ml GM-CSF. Media was then changed to RPMI/1% FCS and the monocyte-derived macrophages (MDMs) left quiescent for 12 hours before incubation with simvastatin 5μM or media control for 4 hours. MDMs were then treated with pooled BALF from either the simvastatin-treated or placebo treated group at 1/10

dilution. After 1 hour the cells were scraped into 1 ml of PBS. Cytoplasmic and nuclear extracts were prepared using a Perbio N-PER nuclear and cytoplasmic extraction kit as previously described (7) and protein content was quantified. Relative concentration of NFkB in 5μg nuclear protein was measured using the TransAM NF-B p65 transcription factor assay kit (Active Motif), according to the manufacturer's instructions (7). Each kit is supplied with a vial of nuclear extract from TPA-activated JURKAT cells, with a final protein concentration of 2.5μg/μl. A relative standard curve was generated using this extract using 2, 1, 0.5 and 0.25 μl of the extract (i.e. 5, 2.5, 1.25 and 0.625 μg protein). The OD_{450} was recorded and using SoftMax Pro software converted to "Relative NFκB units" where 1 relative NFκB unit is equivalent to that found in 1μl of the JURKAT extract. The same JURKAT extract at same dilutions was used for all assays.

In vitro studies to examine the effect of simvastatin pre-treatment on LPS-induced MMP secretion by neutrophils, monocyte-derived macrophages (MDMs) and alveolar epithelial cells

In keeping with the MMPs found to be significantly modified by simvastatin *in vivo* we measured MMP-7/-8 and -9. Neutrophils secrete MMP-8/-9, MDMs secrete MMP-7/-9 and epithelial cells secrete MMP-7/-9.

Methods

Neutrophils

Peripheral blood neutrophils were isolated from 3 donors by dextran sedimentation followed by centrifugation across a Ficoll Paque gradient as previously described (8).

Cells were resuspended in KRG medium with 1% FCS at density of $1x10^6$ per ml in 250μl medium. Cells were either pre-treated with simvastatin (1μM) or an equivalent volume of vehicle (DMSO) for 4 hours before stimulation with LPS 100ng/ml. Culture medium was collected at 4 hours, and centrifuged at 500g to obtain cell free suspension.

MDMs

MDMs were matured as described in the main manuscript. Monocytes from 3 separate volunteers were seeded at $3x10^5$/well of 24 well plate. MDMs were pre-treated for 4 hours with simvastatin 1μM before stimulation with LPS 100ng/ml. Supernatants were collected at 24 hours.

Epithelial cells

Human alveolar type II (hATII) cells from a single donor were seeded at $5x10^5$/well in DCCM and stimulated at 70% confluence as previously described (9). The hATII cells were isolated from patients undergoing lung resection for lung cancer. Cells were isolated from lung remote from the tumor. In addition only subjects with lung function within the normal range were used. Phenotypic characteristics of the hATII cells were confirmed by alkaline phosphatase expression. The presence of mRNA transcripts for surfactant protein C confirmed type II characteristics, whereas a type I cell phenotype was excluded by negative expression for receptor for advanced glycation endproducts and aquaporin 5. Average purity was 92%.

A549 cells were seeded in 24 well plates at $1x10^5$/well and grown in DMEM until 70% confluent. Medium was changed to 1%FCS DMEM/DCCM prior to simulation. Cells were pre-treated for 24 hours with simvastatin 1μM before stimulating with LPS100ng/ml. Supernatants were collected at 72 hours.

MMPs from each cell as described above were analysed by cytometric bead array (R&D Systems, Europe) in cell free culture media as described in the main manuscript.

Results

Neutrophils

LPS stimulation did not increase MMP-8 secretion and simvastatin had no effect (Figure 1a). LPS tended to increase MMP-9 secretion by neutrophils but this was not statistically significance. Simvastatin had no effect on MMP-9 secretion (Figure 1b).

Data are expressed as a function of unstimulated PMN MMP-8/-9 secretion (since there is wide inter-individual variation in baseline and induced MMP secretion due to promoter polymorphisms) and are non-parametric.

MDMs

LPS increased secretion of MMP-7 and -9 from MDMS. While simvastatin tended to reduce secretion of both MMPs this was only statistically significant for MMP-9 (Figure 2). Data are presented as function of control to allow for the large baseline and induced variations between donors for individual MMPs. Of note when raw data, without normalisation to control, were analysed the relationships were the same with a significant reduction in MMP-9 only in the simvastatin-treated group. Data for MMP-7 are non-parametric while data for MMP-9 are parametric.

Epithelial cells

In hATII cells MMP-7 was upregulated in response to LPS. Simvastatin had no effect on LPS-induced MMP-7 secretion (Figure 3a). LPS or simvastatin had no effect on MMP-9 secretion by hATII cells (Figure 3b). Data are parametric.

For A549 cells only MMP-7 was detectable which was not increased in response to LPS (data not shown).

Adverse event reporting

Adverse events were defined according to the ICH Guideline on Good Clinical Practice and in keeping with UK Medicines for Human Use (Clinical Trials) Regulations 2004.

The schedule for patient safety monitoring is given in Table E1

Clinically important muscle disease with regards to statins is defined in most studies as muscle pain with creatine phosphokinase (CPK) levels greater than 10 times the upper level of normal (10).

Subjects were advised to report unexplained muscle pain, tenderness, or weakness. Symptoms of myopathy or an increase in CPK greater than 5 times the upper limit of normal (which was 140 U/L) was defined as an adverse event. Two subjects had an asymptomatic increase in CPK on day 4; 1 in the placebo group (CPK 862 U/L) and 1 in the simvastatin 80mg group (CPK 1015 U/L). At follow-up the CPK level in both subjects was normal. Their family doctor was informed.

An asymptomatic increase in CPK greater than 10 times the upper limit of normal or an increase in CPK greater than 5 times the upper limit of normal associated with symptoms was defined as a serious adverse event. This did not occur in any subject.

In relation to liver toxicity, an increase in alanine aminotransferase (ALT) or aspartate aminotransferase (AST) greater than 3 times the upper limit of normal was defined as an adverse event. This did not occur in any subject.

On the day of LPS inhalation and bronchoscopy and BAL, participants were observed from the time after LPS inhalation until bronchoscopy and BAL was undertaken in the respiratory outpatient clinic.
Criteria were predefined such that if pulmonary function fell by greater than 10% from baseline, bronchoscopy and BAL would not be undertaken. This did not occur in any subject.

Subjects were monitored during and for 2 hours after the bronchoscopy and BAL. In keeping with standard clinical practice, subjects routinely received supplemental oxygen at 2L/min during bronchoscopy and BAL. Criteria were predefined such that if oxygen saturation measured by pulse oximetry fell to <93%, bronchoscopy and BAL would be stopped. This did not occur in any subject.

Following discharge subjects were advised that they can experience cough and fever within 24 hours following LPS inhalation or bronchoscopy and BAL. Subjects were advised how to manage symptoms (paracetamol as anti-pyretic and analgesia) and given a telephone number to contact a member of the study team if any symptoms developed after leaving hospital. Subjects were reviewed at 24 hours after LPS inhalational.

Three subjects complained of symptoms (cough or chest discomfort) and 5 subjects had a greater than 10% (but less than 15%) fall in FEV1 the day after bronchoscopy and BAL which returned to normal on follow-up. There was no difference between the 2

groups and overall there were no significant changes in FEV1 and FVC between the groups.

As the aim of LPS inhalational was to induce both a pulmonary and systemic inflammatory response, an asymptomatic increase in peripheral WCC and CRP was not reported as an adverse event.

An independent Data Monitoring and Ethics Committee was also appointed to monitor the safety of the study and review adverse events and identified no safety issues.

References

E1. Sohy, C., F. Pons, A. Casset, M. P. Chesnard, F. Lieutier-Colas, P. Meyer, G. Pauli, and F. de Blay. Low-dose endotoxin in allergic asthmatics: effect on bronchial and inflammatory response to cat allergen. Clin Exp Allergy 2006;36(6):795-802.

E2. Michel O, Nagy A, Schroeven M, Duchateau J, Neve J, Fondu P, Sergysels R. Dose-response relationship to inhaled endotoxin in normal subjects. Am J Respir Crit Care Med 1997;156:1157-64.

E3. Maris NA, de Vos AF, Bresser P, van der Zee JS, Meijers JC, Lijnen HR, Levi M, Jansen HM, van der Poll T. Activation of coagulation and inhibition of fibrinolysis in the lung after inhalation of lipopolysaccharide by healthy volunteers. Thromb Haemost 2005;93(6):1036-40.

E4. Report of ERS Task Force: guidelines for measurement of acellular components and recommendations for standardization of bronchoalveolar lavage (BAL). *In* P. L. Haslam and R. P. Baughman, editors. Eur Respir Rev 1999;25-157.

E5. Zhao JJ, Xie IH, Yang AY, Roadcap BA, Rogers JD. Quantitation of simvastatin and its beta-hydroxy acid in human plasma by liquid-liquid cartridge extraction and liquid chromatography/tandem mass spectrometry. *J Mass Spectrom* 2000;35:1133–43

E6. Watt AP, Brown V, Courtney J, Kelly M, Garske L, Elborn JS, Ennis M. Neutrophil apoptosis, proinflammatory mediators and cell counts in bronchiectasis. Thorax 2004;59(3):231-6.

E7. O'Kane CM, Boyle JJ, Horncastle DE, Elkington PT, Friedland JS. Monocyte-dependent fibroblast CXCL8 secretion occurs in tuberculosis and limits survival of mycobacteria within macrophages. J Immunol 2007;178(6):3767-76.

E8. Jenei V, Deevi RK, Adams CA, Axelsson L, Hirst DG, Andersson T, Dib K. Nitric oxide produced in response to engagement of beta2 integrins on human neutrophils activates the monomeric GTPases Rap1 and Rap2 and promotes adhesion. J Biol Chem 2006;281(46):35008-20.

E9. Armstrong L, Medford AR, Uppington KM, Robertson J, Witherden IR, Tetley TD, Millar AB. Expression of functional toll-like receptor-2 and -4 on alveolar epithelial cells. Am J Respir Cell Mol Biol 2004;31(2):241-5.

E10. Thompson PD, Clarkson P, Karas RH. Statin-associated myopathy. JAMA 2003;289(13):1681-90.

Figure legends for online supplement

Figure E1

Neutrophil secretion of (a) MMP-8 and (b) MMP-9. LPS 100ng/ml did not induce secretion of either MMP-8 or -9. Simvastatin (1μM) had no further effect.

Figure E2.

MDM secretion of (a) MMP-7 and (b) MMP-9. LPS induced secretion of both enzymes. Simvastatin (1μM) caused a non-significant reduction in MMP-7 secretion (p=0.13) and reduced LPS-induced secretion of MMP-9. *p<0.01 vs control; # p=0.03 for LPS vs LPS+simvastatin.

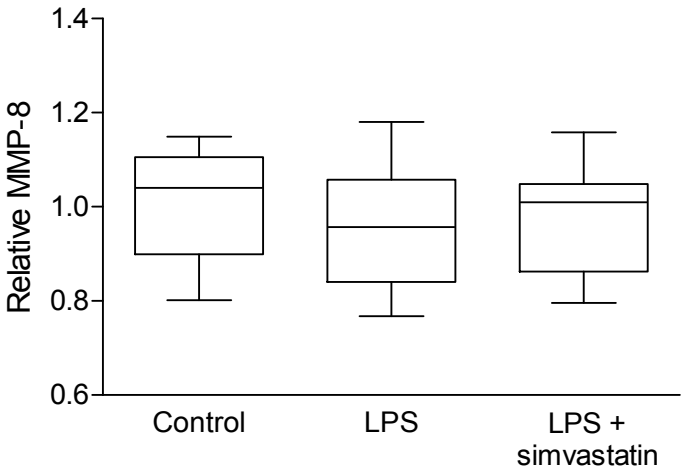
Figure E3.

hATII MMP secretion of (a) MMP-7 and (b) MMP-9. LPS 100ng/ml induced secretion of MMP-7 but not MMP-9 from hATII cells. Simvastatin (1μM) had no further effect. *p<0.001 vs control.

Table E1. Schedule for patient safety monitoring

TIME	Baseline	Day 4	Day 4 Following LPS inhalation	Day 4 Prior to bronchoscopy	Day 5 24 hrs after LPS inhalational
Symptom assessment	*	*	*	*	*
Vital signs and pulse oximetry	*	*		*	*
Routine haematology and renal function	*	*			*
Creatinine kinase (CK)	*	*			*
Liver function tests	*	*			*
Lung function (FEV1 and FVC)	*	*		*	*
Adverse event assessment		*	*	*	*

Figure E1
(a)


1.4
1.2
1.0
0.8
0.6
Relative MMP-8
Control
LPS
LPS +
simvastatin

(b)

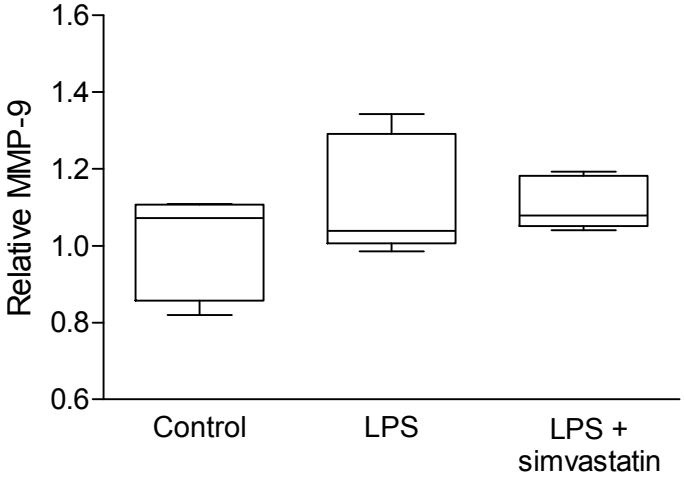
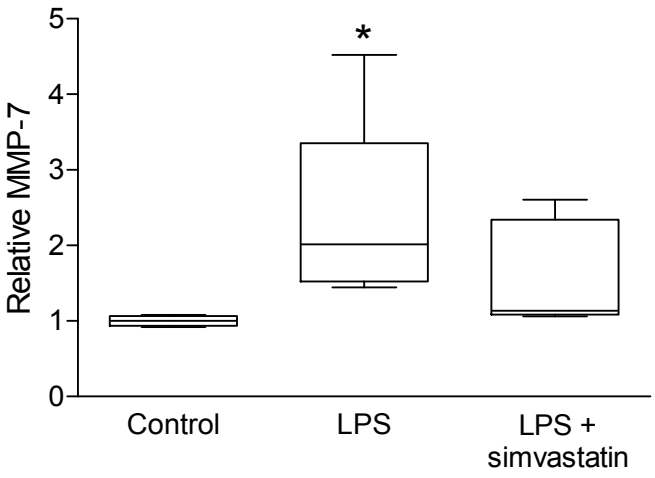

1.6
1.4
1.2
1.0
0.8
0.6
Relative MMP-9
Control
LPS
LPS +
simvastatin

Figure E2
(a)


Relative MMP-7
5
4
3
2
1
0
*
Control
LPS
LPS +
simvastatin

(b)

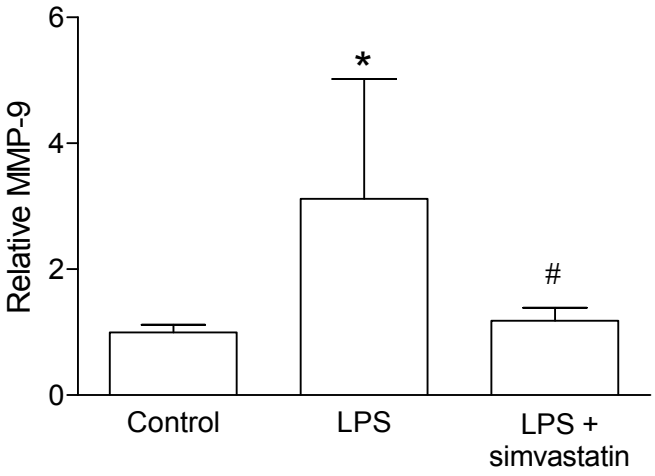
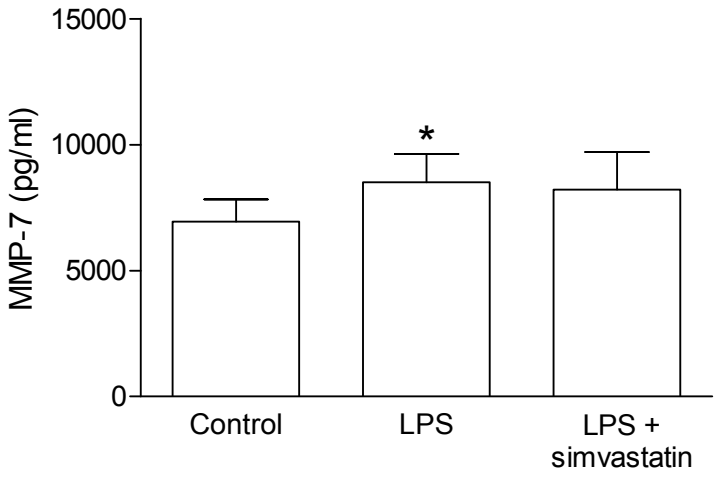
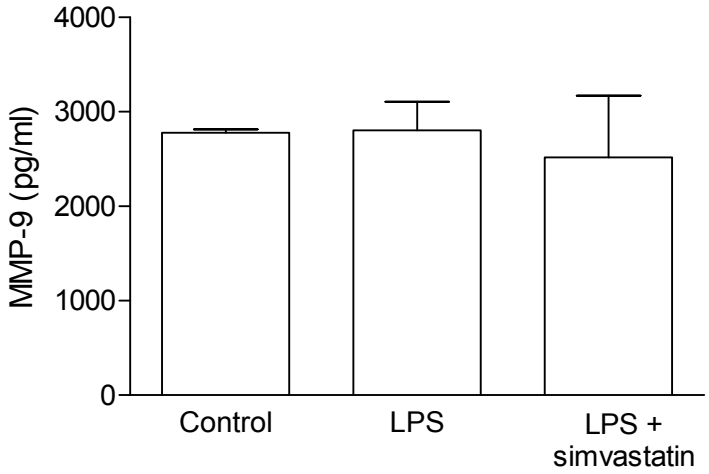

Relative MMP-9
6
4
2
0
*
#
Control
LPS
LPS +
simvastatin

Figure E3
(a)


15000
10000
5000
0
MMP-7 (pg/ml)
*
Control
LPS
LPS +
simvastatin

(b)


4000
3000
2000
1000
0
MMP-9 (pg/ml)
Control
LPS
LPS +
simvastatin